Relations between Phage Sensitivity and Virulence in *Pseudomonas morsprunorum*

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SUMMARY

The phage reactions and pathogenicity of English isolates of *Pseudomonas morsprunorum* were compared with those of isolates from other countries. Non-indigenous cherry strains resembled English cherry strains pathologically, but not all were sensitive to A7, the type-determining phage for English cherry strains. Plum strains resistant to A7 adsorbed the phage as readily as cherry strains, indicating that phage receptor materials are not involved in host specificity. Cherry strain mutants resistant to specific and/or non-specific phages showed marked attenuation of virulence, although retaining the essential pathological characters of cherry strains. The resistance of all but one of the mutants was due to failure to adsorb the homologous phage, showing that the wall structure had changed and suggesting that wall components may function as general virulence determinants in *P. morsprunorum*.

INTRODUCTION

Pseudomonas morsprunorum causes bacterial canker of stone-fruit trees and is most important in England on the cherry (*Prunus avium*) and the plum (*Prunus domestica*). Amongst English isolates of this organism there is a strong correlation between phage sensitivity and the occurrence of virulence characters responsible for host specificity. Thus cherry isolates that are sensitive to A7 and related phages will readily infect cherry trees through leaf scars. Plum isolates, with rare exceptions insensitive to these phages, are non-virulent through cherry leaf scars; furthermore, when mixed in the same inoculum they inhibit infection by the homologous cherry isolates (Crosse & Garrett, 1963; Garrett & Crosse, 1963; Crosse & Garrett, 1970). In this paper we examine the relationship between phage sensitivity and virulence in a selection of non-indigenous *P. morsprunorum* cherry isolates from widely separated geographical localities and describe the effects of mutation to phage resistance on the virulence of indigenous cherry isolates.

METHODS

Bacteria. Details of cultures are shown in Table 1. They were maintained at 4 °C on nutrient agar plus 2 % (w/v) glycerol (NGA). Bacteria were reisolated from diseased tissues on nutrient agar plus 5 % sucrose (NSA). *Pseudomonas morsprunorum* can be readily identified on this medium by colony characteristics (Crosse, 1959).

Phages. These were originally isolated from soils; A1, A3, A7, A9 and A32 with cherry strains of *Pseudomonas morsprunorum* as the propagating species and A15, S5 and S6

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Table 1. *List of isolates of Pseudomonas morsprunorum*

Isolate	Date of isolation	Host	Country of origin
C12	1958	Cherry	England
C9, C22, C46	1961	Cherry	England
C12a*	1965	Cherry	England
C22a*	1966	Cherry	England
C46a*	1968	Cherry	England
C64, C65	1963	Cherry	Holland
C98, C100, C105	1962-3	Cherry	Italy
C167, C169	1965	Cherry	Canada
C170	1965	Cherry	Canada
K1, K2, K3	1962	Cherry	Greece
D10	1960	Plum	England
D17	1961	Plum	England

* Isolates suffixed 'a' were re-isolated from experimental lesions resulting from inoculation with the isolates C12, C22 or C46.

All isolates were confirmed as *P. morsprunorum* by biochemical tests described by Garrett, Panagopoulos & Crosse (1966) for distinguishing this organism from the related pathogen *Pseudomonas syringae*.

with *P. syringae*. Phages of the A7 group (A1 to A9 inclusive and A32) are highly specific for cherry isolates of *P. morsprunorum* (Crosse & Garrett, 1963).

Phage typing. Phages were tested at routine test dilution (RTD) using the procedures and media (phage agar and phage broth) described by Crosse & Garrett (1963).

Phage adsorption tests. Water suspensions of bacteria were prepared from NGA slopes and diluted to about 10^8 organisms/ml with phage broth. To 0.5 ml suspension was added 0.5 ml of phage broth containing the test phage at about 10^8 p.f.u./ml. The mixture was kept at 25 °C for 30 min then 0.1 ml was withdrawn and diluted into 10 ml phage broth containing 0.1 ml chloroform. The mixture was vigorously shaken to kill the bacteria and then assayed for unadsorbed phage by a modification of the Miles & Misra (1938) technique. Serial dilutions of the adsorption mixture were spotted on plates of phage agar seeded with a sensitive indicator strain of *Pseudomonas morsprunorum* and plaques counted after over-night incubation at 25 °C.

Selection of phage-resistant mutants. A suspension in phage broth containing about 10^8 sensitive bacteria/ml and about 10^8 p.f.u./ml of phage was spread on the surface of a phage agar plate. Bacterial colonies appearing after 4 to 7 days at 25 °C were picked-off and streaked on NSA. The process was repeated several times to ensure freedom from extrinsic phage and the final isolate was then re-tested to confirm resistance to the homologous phage.

Plant inoculations. Inoculum was prepared by quickly suspending in sterile distilled water the bacterial growth from 24 or 48 h cultures on NGA slopes. Suspensions were adjusted to the required concentration and then inoculated into 2-, 3- or 4-year-old branches of the cherry cultivars (cv.) Napoleon or Schrecken. Branches were inoculated through leaf scars on fruiting spurs in October or through scalpel wounds in December or January. Treatments were replicated on trees on a random basis (Crosse & Garrett, 1966) and results recorded in the following summer, 5 to 7 months after inoculation.

Disease from leaf scar inoculations was assessed as (i) percentage of diseased spurs, (ii) canker size, and (iii) rate of leaf scar infection. Cankers were graded on an arbitrary rating scale of 1 to 5 inclusive and rate of leaf scar infection, a measure of the infectivity of the bacteria via this route, was calculated as the product (percentage of diseased spurs) $\times$ (mean

Table 2. *Phage sensitivity of isolates of Pseudomonas morsprunorum*

Isolate	Origin	Lysis by phage at RTD					Adsorption of phage A7
		A3, A7	A9	A32	A15	S5, S6	
From cherry:							
C12	England	+	+	+	+	+	+
C22	England						
C65	Holland						
C167	Canada						
C169	Canada						
C170	Canada						
C9	England	+	+	—	+	+	+
C46	England	+	+	+	—	+	+
C100	Italy						
C105	Italy						
C98	Italy	+	—	+	—	+	+
C64	Holland	—	—	+	—	+	—
K1	Greece	—	—	—	+	—	+
K2	Greece						
K3	Greece						
From plum:							
D10*	England	—	—	—	+	+	+
D17†	England	+	+	+	+	+	+

* Typical of the majority of English isolates from plum.

† A rare example, previously noted (Crosse & Garrett, 1970), of a plum isolate of cherry phage type.

canker size) (Crosse & Garrett, 1970). Results were compared by analysis of variance using an angular transformation for percentage of diseased spurs and a square root transformation for rate of leaf scar infection. Disease from wound inoculations was assessed by recording the number of successful infections and the length of the resulting cankers.

RESULTS

Comparison of indigenous and non-indigenous (NI) isolates

Phage typing and phage adsorption (Table 2). The phage reactions of C9, C12, C22 and C46 were typical of English cherry strains; all those so far examined were sensitive to phages A3 and A7 and most of them sensitive to the related phages A9 and A32 (Crosse & Garrett, 1963; Crosse & Garrett, 1970). The reactions of D10, insensitive to A3, A7, A9 and A32 but sensitive to A15, S5 and S6, were similarly characteristic of most English plum strains (Lazar & Crosse, 1969; Crosse & Garrett, 1970).

Seven of the NI cherry isolates were of similar phage type to English cherry strains (Canada: C167, C169, C170; Italy: C98, C100, C105; Holland: C65). The remaining four were insensitive to phages A3, A7 and A9 and in this respect resembled plum strains. They differed however from typical plum strains, e.g. D10, in their insensitivity to S5 and S6 (Greece: K1, K2, K3) or to phage A15 (Holland: C64).

In the phage adsorption tests free-phage titre decreased progressively and after 30 min from 75 to 98 % of phages were adsorbed irreversibly. With the exception of Holland C64, all the isolates adsorbed the cherry-specific phage A7, irrespective of whether they were lysed by this phage or not. The adsorption of A7 by the plum isolate D10 has been repeated in tests with other plum isolates.

Table 3. *Comparative virulence of Pseudomonas morsprunorum isolates in leaf scar and wound inoculations of cherry (cv. Napoleon)*

Leaf scars of fruiting spurs (60 to 100 spurs/isolate) were inoculated on 12 October 1967 with 10^8 bacteria/ml. Wounds (20 replicates/isolate) were inoculated on 15 December with 10^7 bacteria/ml. Water was applied as a treatment, to estimate the background infection from 'wild' strains. Results were scored the following June.

Isolates		Leaf scar infection			Wound infection: canker length (mm)
		Diseased spurs* (%)	Mean canker size rating (max. 5)	Rate of infection (sq. root)	
Origin	No.				
Cherry strains					
England	C9	79.3 (97)	3.77	18.8	117
Holland	C65	80.6 (97)	2.93	16.5	76
Canada	C169	72.1 (91)	2.49	14.8	56
	C170	78.7 (96)	2.39	14.7	52
Greece	K1	66.1 (84)	2.55	14.5	40
Canada	C167	68.8 (87)	2.35	14.0	51
Holland	C64	60.0 (75)	1.96	12.0	42
Greece	K2	60.1 (75)	1.90	11.8	38
	K3	46.1 (46)	1.92	8.8	32
Italy	C98	26.9 (20)	2.28	6.7	40
	C100	31.5 (27)	1.71	6.7	25
	C105	27.4 (25)	1.58	6.1	20
Plum strain					
England	D10	16.5 (8)	0.97	3.1	86
Water controls		18.5 (10)	2.04	5.6	0
Lowest significant differences					
$p = 0.5$		13.33	0.59	2.04	—
$p = 0.01$		17.74	0.78	2.72	—
$p = 0.001$		23.07	2.72	3.54	—

* Back-transformed values are shown to the nearest whole number in parentheses.

Pathogenicity tests. In leaf scar infections all the NI isolates gave significantly lower values for canker size and rate of leaf scar infection than the English reference strain, C9; the majority caused fewer diseased spurs than C9 (Table 3). The NI isolates showed a wide range of virulence, the differences between them for all three disease parameters being frequently significant at the $p = 0.001$ level. The three Italian isolates, C98, C100 and C105, were the least virulent and results for these isolates did not differ significantly from 'wild'-strain infection in the controls. There was no evidence of leaf scar infection with the plum reference strain, D10. Comparison with the controls suggests on the contrary that it reduced natural infection from 'wild' cherry strains present on the leaf surfaces of the experimental trees. This agreed with previous results (Crosse & Garrett, 1970).

All isolates consistently infected through wounds in branches, including D10 which produced longer cankers than any of the NI cherry isolates (Table 3). The length of branch cankers, a measure of the invasive capacity of the isolates in the bark parenchyma, was positively correlated with rate of leaf scar infection ($r = 0.6067$; $p = 0.05$). Only the plum strain, D10, departed significantly from the regression line ($p = 0.02$, Fig. 1). When cherry isolates were considered alone the correlation was considerably improved ($r = 0.8923$; $p = 0.001$) and there was also a correlation between canker length and percentage diseased

Fig. 1. Relation between length of branch cankers and rate of leaf scar infection of cherry (cv. Napoleon) by *Pseudomonas morsprunorum* cherry strains compared with a typical plum strain, D10. The graph is of the form $y = 5.11x - 12.88$.

spurs ($r = 0.6727$; $p = 0.05$). The cherry isolates, irrespective of their origin and phage reactions (Table 2) therefore formed a pathologically similar group distinct from the plum strain, D10. The correlation observed in cherry isolates between leaf scar infectivity and invasive capacity suggest that common virulence factors are involved in both processes.

The effect of mutation to phage resistance on virulence of English cherry isolates

Phage typing and phage adsorption. The phage reactions of the cherry isolates and of their phage-resistant mutants are shown in Table 4. Resistance of mutants was due to failure of the phage to adsorb, except in the case of c22a/s6R which adsorbed the homologous phage, S6, at approximately the same rate as its parent strain. Mutants selected for resistance to any one of the A7 phage group (A1, A7, A9) were resistant to all phages of the group. With one exception, c22/A7R, they were also resistant to the heterologous phage A15. Mutants selected for resistance to A15 or S6, on the other hand, were resistant to the homologous phage only. Apart from phage sensitivity no obvious cultural differences have been observed between mutants and parents. All mutants produced smooth colonies

Table 4. *Phage types of Pseudomonas morsprunorum isolates and their selected phage-resistant mutants*

Isolate; mutant	Lysis by phage at RTD					Adsorption of homologous phage
	A1*	A7*	A9*	A15	S6	
C9	+	+	+	+	+	
C9/A15R	+	+	+	—	+	—
C12, C12a	+	+	+	+	+	
C12/A9R	—	—	—	—	+	—
C12a/A15R	+	+	+	—	+	—
C22, C22a	+	+	+	+	+	
C22/A7R	—	—	—	+	+	—
C22a/S6R	+	+	+	+	—	+
C46, C46a	+	+	+	—	+	
C46/A1R	—	—	—	—	+	—
D17†	+	+	+	+	+	
D17/A1R	—	—	—	—	+	—
Plum strains	—	—	—	+	+	

* Phages specific for *P. morsprunorum* cherry strains.

† A rare example, previously noted (Crosse & Garrett, 1970), of a plum isolate of cherry phage type.

Table 5. *Comparative virulence of Pseudomonas morsprunorum isolates with phage-resistant mutants in leaf scar and wound inoculations of cherry (cv. Napoleon)*

Leaf scars of fruiting spurs (110 to 145 spurs/isolate) were inoculated on 10 October 1965 with 10^6 bacteria/ml. Wounds (18 replicates/isolate) were inoculated on 16 December with 10^7 bacteria/ml. Water was applied as a treatment to estimate background infection from 'wild' strains.

Isolate; mutant	Leaf scar infection			Wound infection: mean canker length (mm)
	Diseased spurs (%)	Mean canker size rating (max. 5)	Rate of infection (sq. root)	
C9	96.1	4.33	20.41	45
C9/A15R	65.9*	2.76*	13.40*	25
C12	95.5	3.29	17.76	20
C12/A9R	25.5*	2.73†	8.25*	25
C22	94.7	4.23	19.97	42
C22/A7R	23.1*	2.59*	7.46*	23
C46	94.6	4.01	19.51	19
C46/A1R	27.5*	3.03†	8.81*	9
D17	91.4	3.42	17.60	21
D17/A1R	32.3*	2.44†	8.79*	17
Water control	20.2	3.09	6.57	—

*, † Mutant differing significantly from the parent strain at $p = 0.001$ and $p = 0.01$ respectively.

on NSA and NGA with growth rates comparable to the parent strain. Some differences in fine colony structure were observed between mutants and parents but these were no greater than those occurring between parent strains. In routine biochemical tests (Garrett, Panagopoulos & Crosse, 1966) mutants and parents were indistinguishable.

Pathogenicity tests. In leaf scar infections of the cherry cultivar Napoleon, mutants showed marked attenuation of virulence compared with the parent strains. The differences

Table 6. *Comparative virulence of Pseudomonas morsprunorum isolates and phage-resistant mutants in leaf scar inoculations of cherry (cv. Schrecken)*

Leaf scars of fruiting spurs (approx. 90 spurs/isolate) were inoculated on 7 October 1971 with 3×10^7 bacteria/ml. Water was applied as a treatment to estimate background infection from 'wild' strains.

Isolate; mutant	Diseased spurs (%)	Mean canker size rating (max. 5)	Rate of infection (sq. root)
C12a	97.5	2.93	16.90
C12/A9R	25.8	1.38	5.97
C12a/A15R	49.5	1.80	9.44
C22a	95.2	2.78	16.27
C22/A7R	23.2	2.00	6.81
C22a/s6R	7.4	1.42	3.24
C46a	95.7	3.33	17.85
C46/A1R	63.7	1.43	9.54
Water control	6.0	1.00	2.45

Fig. 2. Rate of cherry (cv. Napoleon) leaf scar infection resulting from different inoculum concentrations of *Pseudomonas morsprunorum* c22 in the presence and absence of a fixed concentration (about 10^7 bacteria/ml) of its phage-resistant mutant, c22/A7R. ●, c22 alone; ○, c22 plus c22/A7R; ---, level for c22/A7R alone; . . . , level of control 'wild' strain infection.

between mutants and parents were significant for all three disease parameters, in most cases at the $p = 0.001$ level (Table 5). Similar results were obtained in a second experiment on the cultivar Schrecken using a different group of isolates (Table 6). Since the differences in Schrecken were at least as great as in Napoleon the data were not analysed. Re-isolations from diseased spurs of Napoleon inoculated with C9/A15R, C22/A7R or C46/A1R yielded 85 %, 46 % and 59 % respectively of isolates of the appropriate phage mutant type. The mutants were therefore stable during host passage, as are parent cherry strains (Crosse & Garrett, 1966). In wound inoculations of Napoleon branches all mutants except C12/A9R caused smaller cankers than the parent strain (Table 5).

The effect on leaf scar infection of adding a phage-resistant mutant to the cherry strain inoculum

Strain C22 inoculated to cherry leaf scars at a range of concentrations gave an expected sigmoid dosage response relationship (Crosse & Garrett, 1970). When the mutant C22/A7R was added to the C22 inocula, at a constant high level, there was no sign of the inhibitory effect associated with the presence of plum strains in the inocula. On the contrary, the rate of infection was significantly increased when the mutant to parent ratio was 100:1 ($p = 0.01$ level) or higher ($p = 0.001$ level) (Fig. 2). The mutant alone caused some infection but values for all three disease parameters were significantly less ($p = 0.001$ level) than for C22 at the same concentration.

DISCUSSION

In previous work with indigenous plum and cherry strains of *Pseudomonas morsprunorum*, only those sensitive to phage A7 (all cherry strains and very rare plum strains such as D17) were infective through cherry leaf scars, suggesting a possible link between phage sensitivity and the pathological characters responsible for host specificity in these organisms (Crosse & Garrett, 1970). This is not supported by the present experiments with phage-resistant mutants and non-indigenous cherry strains. A significant proportion of these were insensitive to A7 yet they behaved in pathological tests as cherry and not as plum strains. There was also no relationship between host specificity in *P. morsprunorum* and adsorption of phage A7. One of the non-indigenous cherry strains, C64, and several of the mutants failed to adsorb A7, while plum strains apparently adsorb it as readily as 'wild' cherry strains.

The failure of plum strains to infect through cherry leaf scars and their inhibitory effect on homologous cherry strains in mixed inocula have been attributed to the induction of a hypersensitivity reaction in cherry tissues (Crosse & Garrett, 1970). The results of the phage adsorption experiments indicate that this reaction is not due to the presence in the walls of plum strains of material acting as specific phage receptor sites. This is confirmed by experiments with other plant pathogenic *Pseudomonas* spp. to be reported later. Several of these showed inhibitory activity similar to plum strains in mixed infection experiments but were unable to adsorb A7. Other work suggests that the factors responsible for the inhibitory effect of plum strains may not be located in the bacterial wall. We have been unable to reproduce the specific inhibitory effect of plum strains with wall preparations, or to induce a hypersensitivity reaction in tobacco leaves with these preparations (Klement, 1963).

The experiments with phage-resistant cherry strain mutants suggest that there may be wall components involved in the pathogenicity of *Pseudomonas morsprunorum* although not in its host specificity. The attenuation of virulence following mutation to phage resistance occurred with resistance to A15 and S6 as well as to the cherry strain specific group of A7 and related phages. All mutants retained the essential pathological characters of cherry strains. Thus, the reduced leaf scar infectivity of mutants was accompanied in most instances by reduced invasive capacity in wound inoculations, indicating a correlation between these two activities similar to that observed for 'wild' cherry strains (Fig. 1). Also, in mixed infection experiments with its parent strain, the mutant C22/A7R showed none of the inhibitory activity associated with the presence of plum strains in the inocula (Fig. 2). It seems clear therefore that the decreased virulence of mutants was due to a change in characters other than those responsible for the host specificity of *P. morsprunorum*. Six

of the seven mutants tested failed to adsorb the homologous phage (Table 4) showing that there had been some change from the parent in phage receptor material in the bacterial wall. Using procedures for extracting lipopolysaccharides from bacterial walls (Westphal & Jann, 1965), we have recently obtained materials that inactivate phages specifically *in vitro*. Keen & Williams (1971) reported the isolation of a lipopolysaccharide complex from a related pathogen, *Pseudomonas lachrymans*, and showed that this induced water soaking in the leaves of its natural host, the cucumber. Hoitink & Bradfute (1972) could not induce disease in tobacco using gymnoplasts of *Pseudomonas tabaci* and suggested therefore that pathogenicity determinants were located in the wall of the pathogen. It is plausible to speculate from our work that the wall of *P. morsprunorum* contains materials which play a role in pathogenicity analogous to the lipopolysaccharide endotoxins of the Enterobacteriaceae. The fact that one of the mutants, c22a/s6R, showed marked attenuation of virulence without any loss of phage adsorptive capacity (Table 4) does not necessarily conflict with this theory, since the phage resistance of this mutant may result from changes in macromolecular wall components affecting phage penetration. We have no evidence of this, however, and the results with other mutants must therefore be interpreted with caution. Changes in wall structure and virulence may be no more than associated characters resulting from some other change in the mutants, i.e. in basic carbohydrate metabolism.

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